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Translational multiple sclerosis research in primates

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Severe oxidative stress in an acute inflammatory demyelinating model in the rhesus monkey

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Abstract

Background: Oxidative stress is increasingly implicated as a co-factor of tissue injury in inflammatory/demyelinating disorders of the central nervous system (CNS), such as multiple sclerosis (MS). While rodent experimental autoimmune encephalomyelitis (EAE) models diverge from human demyelinating disorders with respect to limited oxidative injury, we observed that in a non-human primate (NHP) model for MS, namely EAE in the common marmoset, key pathological features of the disease were recapitulated, including oxidative tissue injury.

Method: Here, we investigated the presence of oxidative injury in another NHP EAE model, i.e. in rhesus macaques, which yields an acute demyelinating disease more closely resembling acute disseminated encephalomyelitis (ADEM) than MS. Rhesus monkey EAE diverges from marmoset EAE by abundant neutrophil recruitment into the CNS and destructive injury to white matter. This difference prompted us to investigate to which extent the oxidative pathway features elicited in MS and marmoset EAE are reflected in the acute rhesus monkey EAE model.

Results: The rhesus EAE brain was characterized by widespread demyelination and active lesions containing numerous phagocytic cells and to a lesser extent T cells. We observed induction of the oxidative stress pathway, including injury, with a predilection of p22phox expression in neutrophils and macrophages/microglia. The oxidative stress and injury could be attributed to the accumulation of iron in the brain.

Conclusions: These results indicate that pathogenic mechanisms in the rhesus EAE model may differ from the marmoset EAE and MS brain due to the neutrophil involvement, but in the end lead to similar induction of oxidative stress and injury.



Introduction

Cell-mediated demyelinating diseases of the central nervous system (CNS) are idiopathic disorders that include multiple sclerosis (MS) and acute disseminated encephalomyelitis (ADEM) ¹. As these diseases have a predilection for onset and diagnosis in children (ADEM) or young adults (MS), the quality of life is adversely affected during prime activity years ². One inherent commonality of these diseases, as well as with other neuro-immunological disorders, such as stroke, is a dysfunction of the blood-brain barrier and leukocyte extravasation from the periphery into the CNS ^{1,3}. Although both the innate and adaptive arms of the immune system play a role in disease activity, the innate immune arm plays a particularly vital role in orchestrating demyelinating pathologies ⁴.

Non-human primates (NHP) have emerged as valuable pre-clinical models for the translation of scientific discoveries in rodent-based models of (auto)immune inflammatory diseases to clinical application ⁵. NHP, compared to mice, not only have a much closer evolutionary proximity to humans, which is reflected by genetic and immunological similarity, but they also display more similar complexity of neuro-anatomical structures ^{6,7}. EAE can readily be induced in diverse NHP species. Both common marmosets (*Callithrix jacchus*) and rhesus macaques (*Macaca mulatta*) are equally susceptible to developing EAE, yet they differ in their requirement for adjuvant in EAE induction and the manner in which the ensuing disease progresses ⁸⁻¹¹. Despite a closer biological and phylogenetic relationship of rhesus monkey to human than marmoset, disease development in the rhesus EAE model is much more acute and aggressive. A characteristic pathological difference between the EAE models in marmoset and rhesus monkeys is the presence of large necrotic/hemorrhagic lesions in the latter, which are absent in the marmoset ⁹. Clinically, EAE in rhesus monkeys resembles ADEM, one of the juvenile forms of MS and displays a rapid and aggressive clinical course expressing symptoms such as vomiting, vision impairment, and acute neurological deficits, such as rapid onset of hemiparesis ^{12,13}.

In both ADEM and rhesus monkey EAE, evidence for a pathogenic role of neutrophils was found, but their exact role in the animal model has not been analysed ¹⁴. Neutrophilic granulocytes are abundant in peripheral blood and rapidly mobilize to sites of infection or damage where they phagocytose pathogens, release proteinases and anti-microbial peptides, and form extracellular traps ^{4,15}. Similar to macrophages, neutrophils generate reactive oxygen species (ROS) by electron chaperone NADPH oxidase complexes localized in phagolysosomes and cell membranes. The activation of NADPH oxidase involves the assembly of cytosolic (p47phox and p67phox) with membrane-bound (gp91phox, p22phox) subunits into a multimeric complex ¹⁶. While ROS have a vital role in the intra-phagosomal degradation of pathogens, they can elicit oxidative stress and oxidative tissue injury when released outside cells ¹⁷. The brain is naturally highly vulnerable to oxidative stress, which is due to the high polyunsaturated fatty acid content of the neuronal membranes and the high oxygen consumption relative to the rest of the body ¹⁸. Oxidative injury and mitochondrial dysfunction are now implicated as main causative factors of axon degeneration in the MS brain (reviewed in ^{19,20}).

The inflammatory active MS lesion is characterized by the expression of ROS generating NADPH oxidase in resident (microglia) and infiltrated (macrophages) phagocytic cells. The

presence of markers associated with oxidative stress coincided with marked upregulation of anti-oxidant enzymes, such as superoxide dismutase (SOD) 1 and 2^{21,22}. SOD2 was prominently expressed in astrocytes and neurons of the MS and marmoset EAE brain^{21,23,24}. Up-regulation of mitochondrial heat shock protein 70 (mtHSP70) is a known tissue reaction to oxidative stress to minimize protein aggregation²⁵. Recently, we have shown that oxidative stress and injury are key pathological features of the marmoset EAE model (Dunham et al. 2017, *J Neuropathol Exp Neurol*, In press). The inadequate replication of these features in rodent EAE models underscores the translational relevance of the marmoset model²⁶. The fundamentally different severity of oxidative stress and white matter injury observed between the marmoset EAE model and rodent EAE models can in part be attributed to the accumulation of iron observed in both marmoset and human brain. Iron may amplify oxidative injury by catalyzing the formation of highly toxic hydroxyl radicals via the Haber-Weiss reaction²⁷.

The aim of the current study was to characterize the oxidative stress pathway in the acute demyelinating rhesus monkey EAE model. We show abundance of NADPH oxidase expressing neutrophils and iron in the rhesus monkey EAE brain. These elements combined underlie the strong oxidative stress and injury that characterize the lesions in this model.

Material and Methods

Rhesus monkey tissues

Cryopreserved and formalin-fixed, paraffin-embedded tissues from previous rhesus monkey EAE experiments performed at the Biomedical Primate Research Centre (BPRC, Rijswijk, The Netherlands) were used for this study. These studies were reviewed and approved by the institutional ethics review committee. EAE induction was performed by immunization with human recombinant myelin oligodendrocyte glycoprotein (rhMOG) emulsified in CFA (rhMOG/CFA). The monkeys for this study were selected based upon development of clinically evident disease severe enough to warrant humane euthanasia. For a detailed description of disease induction and monitoring see Haanstra et al¹².

Immunohistochemistry

Immunohistochemical analysis of formalin-fixed paraffin embedded material was performed on 5 µm sections. Sections were deparaffinized using xylene (VWR, Radnor, PA), rehydrated via graded ethanol into distilled water and blocked for endogenous peroxidase activity by incubating tissue in 0.03% hydrogen peroxide (Sigma, St. Louis, MO,) in methanol for 30 min. Heat-induced antigen retrieval was performed in either EDTA (pH 8.6; Sigma) or citrate (pH 6.0; Sigma) and tissue was rinsed twice in Tris-buffered saline (TBS) following steaming. To block non-specific antibody binding, tissue sections were incubated in 10% FCS in Dako wash buffer (DAKO, Glostrup, Denmark) for 30 min. To stain cryo-preserved material, sections of 6-8 µm were mounted onto permafrost plus tissue slides and fixated with acetone (10 min). Prior to nonspecific antibody blocking tissue sections were air dried for 10 min, rinsed with PBS, and incubated in PBS with 0.03% hydrogen peroxide to block endogenous peroxidase.



Tissue sections were incubated with primary antibodies (See **Table 1**) overnight at 4°C. Following wash steps with TBS to remove excess antibody. Biotin-labeled secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) were added to tissue sections for 1 h at room temperature. Following an additional wash step, avidin-labeled peroxidase (Sigma, 1:150) was added prior to visualization with diaminobenzidine tetrachloride (DAB, Sigma). A hemalaun counterstain was performed to visualize nuclei by incubation tissue for 2 min in a 1:10 diluted hemalaun (Merck Millipore; Billerica, MA). Finally, tissue was dehydrated with graded ethanol and xylene prior to mounting with malinol (Waldeck, Münster, Germany).

To quantify expression of cellular and oxidative stress markers, 400x images of normal appearing white matter (NAWM) (5 animals) and of active lesions (5 animals) were converted to 8 bit using ImageJ and a threshold was applied (to images) to eliminate non-specific staining. Standard scale bars were used to calculate the area of the images to determine cells/area and data is presented as the number of positively staining cells /mm².

Table 1. Antibodies used for immunocytochemistry

| Primary antibody | Company | Catalog | Host | Target |
|------------------|----------------------|-------------|--------|---------------------------------|
| HLA-DR | DAKO | M0775 | Mouse | Antigen presentation |
| Iba-1 | Abcam | AB15690 | Mouse | Microglia |
| MRP14 | BMA biomedicals | S100A9 | Mouse | Macrophage |
| CD3 | Agilent Technologies | A0452 | Rabbit | T cell |
| CD66 | Miltenyi Biotec | 130-093-133 | Mouse | Neutrophil |
| PLP | Serotec | MCA839G | Mouse | Myelin |
| GFAP | Sigma | SAB5201104 | Mouse | Astrocyte |
| NeuN | Millipore | MAB377 | Mouse | Neuron |
| P22phox | Santa-cruz | SC20781 | Rabbit | NADPH oxidase subunit |
| iNOS | Chemicon | AB16311 | Rabbit | Reactive nitrogen species |
| mtHSP70 | ThermoFisher | MA3-028 | Mouse | Oxidative stress (mitochondria) |
| SOD2 | Abcam | ab13533 | Rabbit | Oxidative Stress (mitochondria) |
| 8-OHdG | Abcam | ab62623 | Mouse | Oxidative Injury |

Immunofluorescence

Double or triple fluorescent labeling was employed to determine co-localization of expression of various markers associated with the oxidative damage pathway. Staining was performed similar to the immunohistochemistry protocol described above, with minor deviations. Briefly, following overnight incubation at 4°C with primary antibodies diluted in Dako ready-to-use diluent (DAKO) or TBS with 10% FCS, slides were washed in TBS and incubated for an additional 1 h. For visualization, tissue was incubated for 1 h at room temperature with directly labeled secondary antibodies against primary host species conjugated to CY2, CY3 and CY5 (Jackson ImmunoResearch Laboratories,). Cell nuclei were visualized using a commercially available Vectashield DAPI antifade mounting kit (Vector laboratories, Burlingame, CA). Fluorescence images were taken with a 63x oil immersion lens on a Leica systems microscope (DMI 600B; Leica Microsystems GmbH, Wetzlar, Germany).

Iron staining

To determine the iron content of the post-mortem primate brain, DAB-enhanced Turnbull staining of non-heme tissue iron was performed on formalin-fixed paraffin-embedded tissue sections as described^{28,29}. Briefly, paraffin-embedded tissue sections of 5 µm thickness were deparaffinized using xylene and then rehydrated via graded ethanol into distilled water. Tissues were treated with 10% ammonium sulfide (Merk Millipore; NY) for 1.5 h and with potassium-ferricyanide (Sigma) for 15 min. Endogenous peroxidase was blocked with 0.03% hydrogen peroxide in methanol (Sigma) prior to amplification with 0.025% 3,3'-diaminobenzidine (Sigma, St. Louis, MO). Hemalaun counterstaining was performed as described above.

Figures and statistics

Figures were made using Adobe InDesign CC 2015 (Adobe Systems, San Jose, CA) and in some cases images were adjusted for brightness using Adobe Photoshop CC 2015 (Adobe Systems). Statistics (Mann-Whitney test) was performed using Graphpad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA).

Results

Neutrophil-mediated widespread demyelination in rhesus monkey EAE

A generic pathological characterization was performed on brains of two non-immunized control rhesus monkeys and five rhesus monkeys with rhMOG/CFA-induced EAE. In the non-EAE animals sparse infiltrates of immune cells such as neutrophils (CD66), macrophages (myeloid-related protein 14 (MRP14)) and T cells (CD3) were observed in NAWM and often small clusters composed of macrophages and neutrophils could be detected in these areas (**Figure 1**). As inflammation and demyelination are widespread, lesion borders of active demyelinating lesions (**Figure 1**) were not as clear as typically observed in marmoset EAE. The cellular infiltrate of active lesions is predominantly composed of neutrophils, macrophages and microglia (**Figure 1D, F, H**). T cells were substantially detected in the active lesion, albeit less frequent in numbers compared to macrophages and neutrophils (**Figure 1J**). Quantification of Iba-1, MRP14, CD66 and CD3 was performed in 15 active lesions and 15 areas of NAWM (**Figure 2**). The number of cells expressing Iba-1, MRP14, CD66 and CD3 were significantly increased in active lesions compared to NAWM, with the highest numbers for macrophages and neutrophils (**Figure 2**).



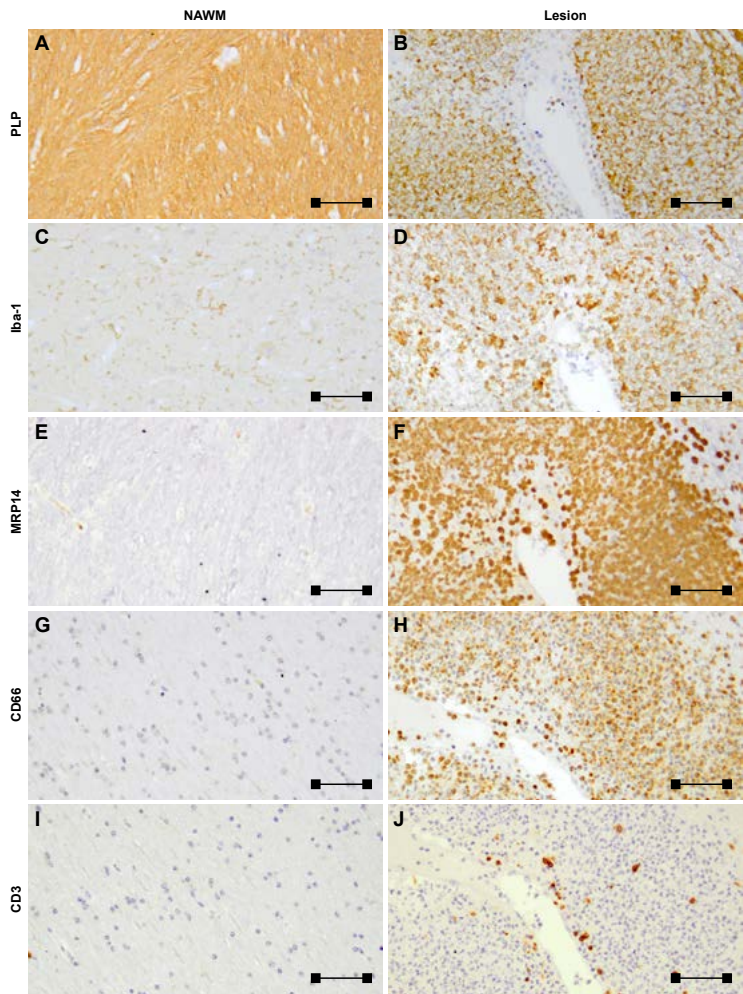


Figure 1. Characterization of rhesus monkey EAE brain pathology. Tissue was stained for myelin (PLP; **A-B**), microglia/macrophages (Iba-1; **C-D**), macrophages & neutrophils (MRP14; **E-F**), neutrophils (CD66; **G-H**), and T cell markers (CD3; **I-J**). In the NAWM (left column), infiltrating immune cells were absent to sparse, yet markedly upregulated in the active lesions (right column). The dominant immune cell types of the active lesion were macrophage/microglia (**D, F**) and neutrophils (**H**). T cells, as determined by CD3 positivity, were readily detected in large numbers, yet visually less abundant than other cell types (**J**). The image scale bar is 100 μ m.

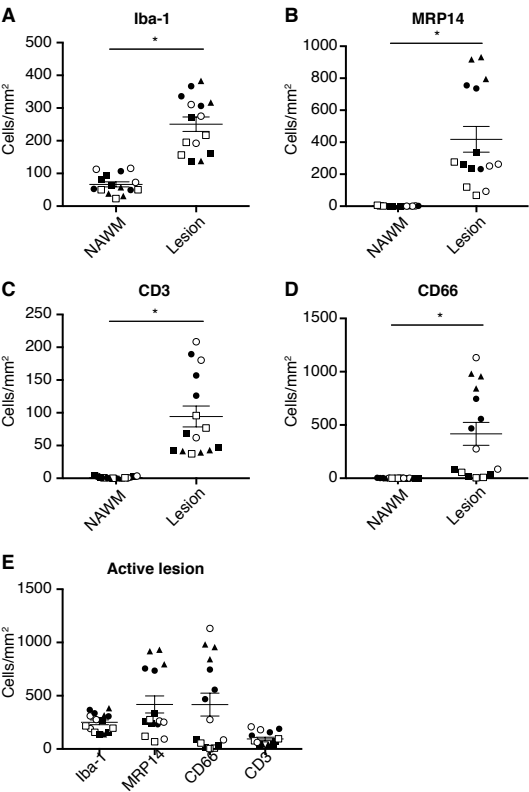


Figure 2. Quantification of lesion cellularity. From 5 monkeys with clinically evident EAE, cell infiltrates in 15 NAWM and 15 active demyelinating areas were quantified with ImageJ and expressed as cells/mm². Shown is the quantification of microglia/macrophage (Iba-1), macrophage and neutrophils (MRP14), neutrophils (CD66) and T cells (CD3) as NAWM versus active lesion (A-D). Lesions of each individual animal are shown by similar symbols, statistics was calculated with the mean per animal. Results were also combined and reported as cells/mm² (E). Statistical significance is indicated as: * when p<0.05.

Accumulation of iron in the rhesus monkey brain

The observation that the different ability of immuno-pathogenic factors in MS and marmoset EAE brain to elicit oxidative tissue injury in comparison to rodent strains may be caused by the lacking accumulation of iron in rodent CNS, prompted us to stain for total non-heme iron (Turnbull staining). Profound iron accumulation could be observed in oligodendrocytes and myelin of the white and grey matter in the control rhesus monkey brain (Figure 3A-C). The diffuse nature of demyelination in rhesus EAE hampered the visualization of pathology-specific alterations of iron accumulation as documented in multi-focal demyelinating patterns in marmoset EAE. Nevertheless, iron loss was clearly observed as a consequence of myelin loss and oligodendrocyte death (Figure 3D-F). This observation indicates that a key factor in the amplification of oxidative injury, observed in both marmoset and human brains, is also present in the rhesus brain.



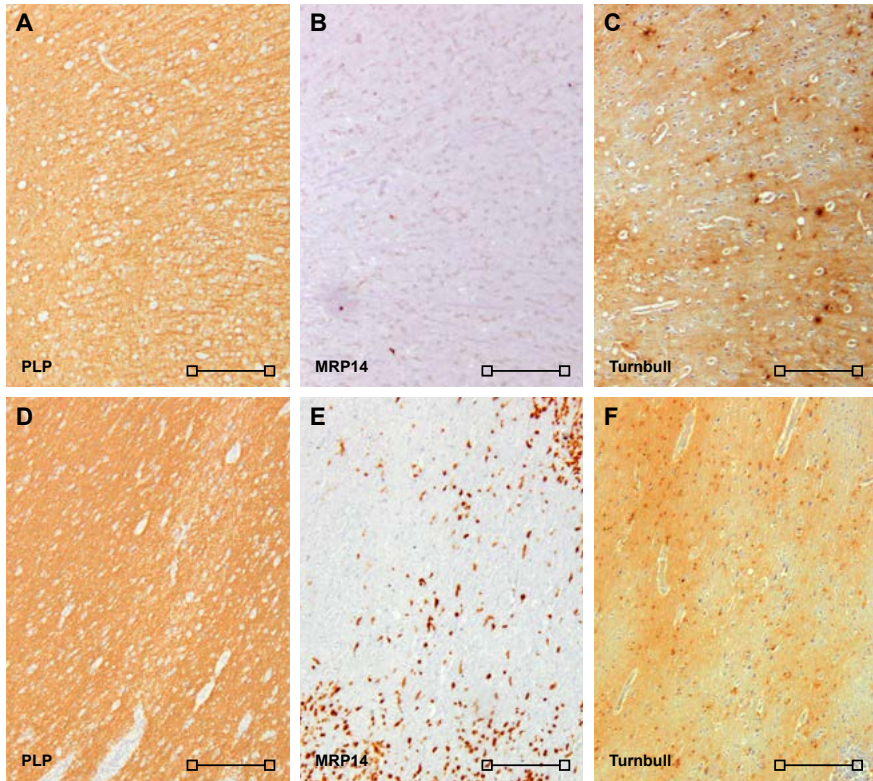


Figure 3. Iron accumulates in rhesus brain. To determine tissue specific iron, Turnbull staining was performed on healthy control and EAE brain tissue from rhesus monkeys. Shown are overview images of PLP (**A,D**), MRP14 (**B,E**), and Turnbull (**C,F**) of a control brain (**A-C**) and an EAE brain (**D-F**). In the control brain, iron accumulation was strongest in the GM (**A,C**). In the EAE brain no distinct pattern of iron staining emerged. Image scale bars are 200 μm (open square).

Marked upregulation of oxidative stress

Next, we examined the presence of oxidative stress in rhesus EAE brain. In NAWM, p22phox, a membrane subunit of NADPH oxidase complex, was sparsely detected, but expression was strongly enhanced in active lesions (**Figure 4A-E, Q**). As expected, expression of p22phox was observed in neutrophils as identified by their classic multi-lobe nucleus appearance, and in mononuclear phagocytes, such as Iba-1+ macrophages and microglia, yet not in astrocytes or other CNS glia cells (**Figure 4L-M**).

Basal expression of mthSP70 was observed in NAWM of the rhesus monkey EAE brain (**Figure 4F, R**) and this was markedly up-regulated in the active lesion (**Figure 4G, R**). Low levels of SOD2 were observed in NAWM (**Figure 4H, S**) of the rhesus monkey EAE brain and this was significantly upregulated in the active lesion (**Figure 4I, S**). Expression was observed in neurons and Iba-1+ macrophages and microglia, but fairly absent in astrocytes (**Figure 4N-P**).

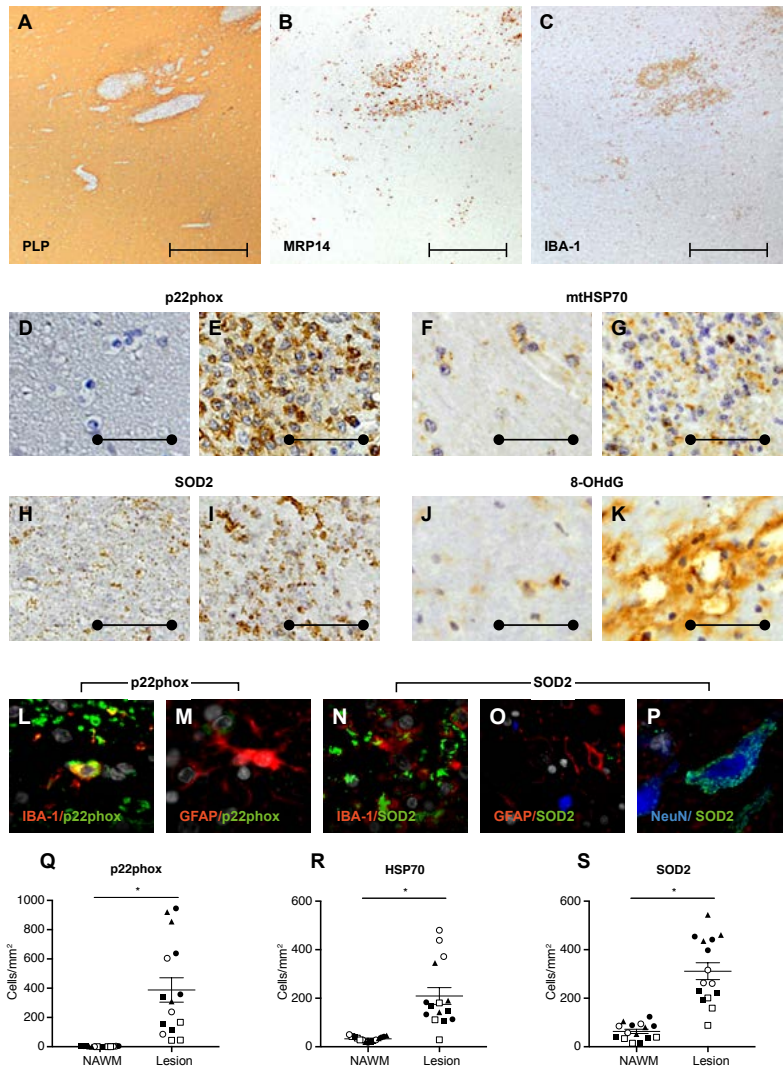


Figure 4. Expression of oxidative stress pathway markers. Key markers of the oxidative stress pathway were analyzed by immunohistochemistry. Shown in figure 4 is a selected brain area with EAE lesions stained for PLP (A), MRP14 (B) and Iba-1 (C). In the NAWM (D), p22phox (D-E, Q) expression ranged from absent to sparse, which contrasted with the marked detection observed in an active lesion (E). Both mtHSP70 (F-G,R) and SOD2 (H-I,S) exhibited basal expression in the NAWM (F, H), while both marker were clearly up-regulated in the active lesion (G,I). Immunoreactivity to 8-OHdG (J-K) was detected in NAWM as small patches (J), yet markedly upregulated in the active lesion (K). Double labeling of select oxidative stress markers was performed depending upon host species of primary antibodies. Expression of p22phox (green) was detected in Iba-1+ (red) macrophages and microglia, yet not in GFAP+ astrocytes or other CNS cells (Fig 4 L,M). Expression of SOD2 (green, N-P) was observed in NeuN+ neurons (blue, P) and Iba-1+ (red, N) microglia/macrophages, but fairly absent in GFAP+ astrocytes (red, O). (Q-S) Lesions of each individual animal are shown by similar symbols, statistics was calculated with the mean per animal. Statistical significance is indicated by * for $p < 0.05$. Image scale bars are 500 μ m (perpendicular line), 50 μ m (closed circle).



Finally, DNA oxidation, a marker for oxidative injury, was assessed by the presence of immunoreactive 8-OHdG. Small focal patches of immunoreactive 8-OHdG were frequently detected in NAWM, while much larger staining areas were found in the active lesion (**Figure 4J-K**). Collectively, this data demonstrates that the oxidative stress pathway is strongly activated in the rhesus monkey EAE brain.

Discussion

Induction of the oxidative stress pathway is a key pathological factor in the progression of MS^{22,30}. Whereas representation of this pathway in rodent EAE models has shown to be very minimal, recent data indicate that this pathway is well represented in the marmoset EAE model (Dunham et al., 2017, *J Neuropathol Exp Neurol* in press). Here we extended these findings to another NHP EAE model, namely in rhesus monkeys. The two models seem to cover the wide spectrum of human autoimmune demyelinating brain diseases, where the chronic EAE model in marmosets resembles MS while the acute EAE model in rhesus monkeys more closely resembles ADEM⁸.

The current histopathological analysis of markers associated with the oxidative stress pathway show that mechanisms associated with the initiation and amplification of oxidative injury are well represented in the acute demyelinating brain lesions in the rhesus monkey EAE model. For both macrophages and neutrophils, the expression of NADPH oxidase and generation of superoxide anions in phagolysosomes play a vital role in pathogen clearance^{31,32}. On the other hand, oxidant production by the NADPH oxidase complex expressed in the cell membrane can be harmful to tissue and result in irreversible oxidative injury. Therefore, healthy tissues are equipped with mechanisms limiting the consequences of oxidative stress (i.e. ROS scavengers, glutathione redox cycling). Suppression of this oxidative cytotoxicity without affecting the ROS-dependent intracellular killing of microorganisms is therapeutically attractive for MS²². Recent clinical successes of dimethyl-fumarate, which activates the nuclear-related factor 2 (Nrf2) anti-oxidant signaling cascade, suggests cessation of oxidative stress to be clinically beneficial³³.

While the rhesus monkey EAE model diverges from both marmoset EAE and human MS with respect to the histopathological profile of CNS infiltrated immune cells, we propose that there is considerable value of this current model in aiding the development of therapeutics directed towards oxidative injury. Despite the acute nature of this model regarding disease progression, the recruitment of immune cells into the CNS elicited strong representation of the oxidative stress pathway. As rhesus EAE would represent the extreme end of acute oxidative stress in relation to CNS demyelination, therapies showing efficacy via anti-oxidant stimulation in this model, would have a good chance at working in MS.

Primate models of autoimmune demyelination demonstrate tremendous heterogeneity with respect to clinical and neuropathological presentation and thus represent a wide spectrum of inflammatory associated demyelination^{8,34}. The observation that entirely different clinical and pathological processes occur in different primate species indicates an important role of innate factors in the clinical and pathological response to immunization^{8,9}. It is tempting to speculate that the heterogeneous disease course in human demyelinating disorders and in primates is a

function of differential activation of innate immune factors, neutrophils for example, and the role that oxidative stress plays⁹. Moreover, tissue defense mechanisms may differ between species, as shown for the complement regulatory factor CD55³⁵. Although the T and B cells remain the center of attention for immune modulatory therapies, selective targeting of the innate immunity may prove to be success as suggested by others³⁶.

Age-associated accumulation of iron in the human brain is thought to play a critical role in the amplification of oxidative damage in neurodegenerative processes via the Haber-Weiss reaction³⁷. Pathology-associated liberation of iron could enhance the oxidative stress elicited by the respiratory bursts of neutrophils and macrophages³⁷. The prominent accumulation of iron in the rhesus monkey brain is in line with earlier observations that iron accumulates in myelin and oligodendrocytes of the marmoset brain (Dunham et al., 2017, *J Neuropathol Exp Neurol* in press). The results reported here are also consistent with reports on iron in the human brain, suggesting that CNS accumulation of iron is a general neurophysiological feature of humans and NHP and represents a critical divergent from rodent species²⁶.

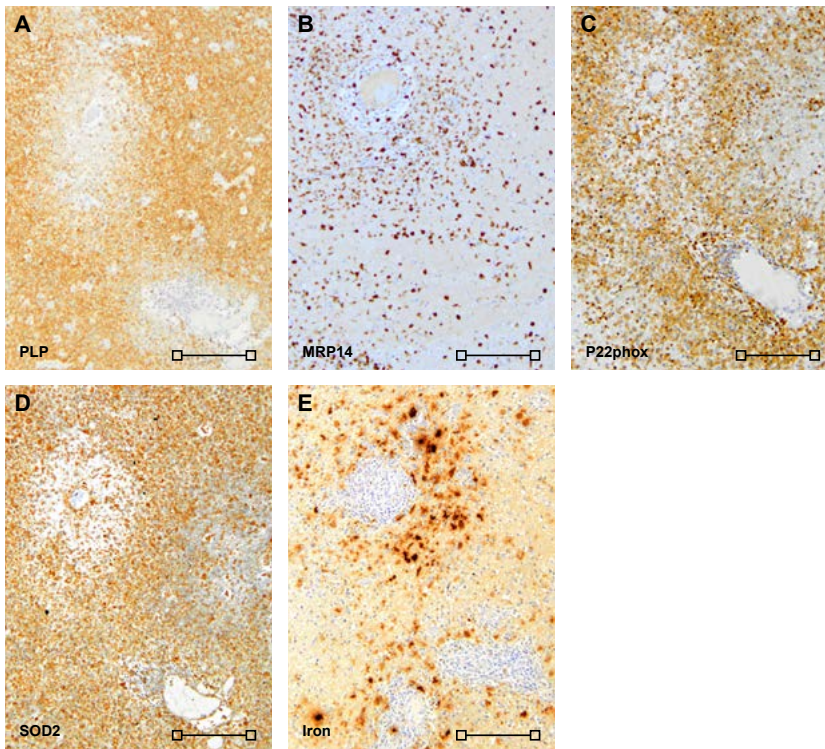
Conclusion

In conclusion, we demonstrate that the rhesus monkey EAE brain, like the marmoset EAE brain, replicates many key features of the oxidative stress pathway observed in human CNS demyelinating disorders. Both NHP EAE models can therefore be beneficial in validating therapy targets for pathogenic mechanisms associated with oxidative injury.

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Supplemental fig 1. Iron and oxidative stress. Shown in supplemental fig 1 are adjacent stains of PLP (A), MRP14 (B), p22phox (C), SOD2 (D) and Iron (E) of an EAE lesion. The image scale bar is 100 μm.

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